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RESEARCH ARTICLE

# Self-reported pregnancy exposures and placental DNA methylation in the MARBLES prospective autism sibling study

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## Abstract

Human placenta is a fetal-derived tissue that offers a unique sample of epigenetic and environmental exposures present in utero. In the MARBLES prospective pregnancy study of high-risk younger siblings of children with autism spectrum disorder (ASD), pregnancy and environmental factors collected by maternal interviews were examined as predictors of placental DNA methylation, including partially methylated domains (PMDs), an embryonic feature of the placental methylome. DNA methylation data from MethylC-seq analysis of 47 placentas of children clinically diagnosed at 3 years with ASD or typical development using standardized assessments were examined in relation to: child's gestational age, birthweight, and diagnosis; maternal pre-pregnancy body mass index, smoking, education, parity, height, prenatal vitamin and folate intake; home ownership; pesticides professionally applied to lawns or gardens or inside homes, pet flea/tick pouches, collars, or soaps/shampoos used in the 3 months prior to or during pregnancy. Sequencing run, order, and coverage, and child race and sex were considered as potential confounders. Akaike information criterion was used to select the most parsimonious among candidate models. Final prediction models used sandwich estimators to produce homoscadisticity-robust estimates of the 95% confidence interval (CI) and *P*-values controlled the false discovery rate at 5%. The strongest, most robust associations were between pesticides professionally applied outside the home and higher average methylation over PMDs [0.45 (95% CI 0.17, 0.72), $P = 0.03$] and a reduced proportion of the genome in PMDs [−0.42 (95% CI − 0.67 to − 0.17), $P = 0.03$]. Pesticide exposures could alter placental DNA methylation more than other factors.

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**Key words:** environmental factors; pregnancy; autism spectrum disorders; pesticides; placenta DNA methylation; high-risk cohort

## Introduction

Autism spectrum disorder (ASD), a neurodevelopmental disorder characterized by impairments in social behavior and communication, as well as restrictive and repetitive interests and behaviors, currently affects an estimated 1 in 68 children in the USA [1]. Emerging evidence suggests that ASDs and other neurodevelopmental disorders arise from complex gene–environment interactions [2–6]. Although several environmental risk factors for ASD have been identified, including prenatal air pollution and pesticide exposure [7], and evidence for gene–environment interactions is emerging [8, 9], additional environmental factors are likely to influence susceptibility to ASD and the mechanisms by which environmental factors interact with genetic factors to determine individual risk remain critical gaps in our understanding of ASD etiologies.

High-risk sibling study designs can aid the identification of factors that contribute to increased risk for ASD in highly susceptible populations and offer a feasible prospective design that can evaluate mechanistic markers. The prospective MARBLES (Markers of Autism Risk in Babies: Learning Early Signs) study recruits mothers of at least one child diagnosed with ASD who are either planning or are pregnant with another child, in order to understand what influences the outcome of the younger high-risk sibling and to identify early markers of ASD. Given the family history of ASD, with shared environmental and genetic factors, the sibling is at higher risk for developing ASD and other adverse neurodevelopmental outcomes than children from the general population. Younger high-risk siblings are affected with ASD at rates of nearly one in five (19%) [10], with significant differences in familial recurrence between males (27%) versus females (10%) [11]. Another 21% of these high-risk younger siblings are affected with other aberrant neurodevelopmental outcomes, including attention problems or language delays, or broader autism phenotype, leaving about 60% with typical development (TD) [10].

Epidemiologic evidence suggests that periconception and *in utero* periods are the most vulnerable to environmental factors influencing ASD risk [7, 12–16]. This study leverages the MARBLES population with increased genetic susceptibility to ASD and other neurodevelopmental disorders to evaluate which gestational factors most influence global placental methylation levels as a potential mechanistic biomarker.

DNA methylation marks could be ideal biomarkers at the interface of ASD genetic and environmental risk factors [17]. Placenta is an accessible tissue with distinctive global and site-specific DNA methylation patterns compared with somatic tissues. Unlike the embryoblast, the trophoblast-derived placenta does not undergo extensive re-methylation following the wave of post-fertilization de-methylation, and, thereby, maintains a genome-wide hypomethylated state [18,19]). Throughout pregnancy, the human placenta maintains a bimodal DNA methylation pattern characterized by interspersed partially methylated domains (PMDs) and highly methylated domains (HMDs) that are detectable in both sequencing and array-based platforms [20]. PMDs are relatively large (over 100 kb) regions that span transcriptionally repressed gene loci enriched for synaptic and ASD candidate genes, together making up ~40% of the placental genome [20, 21]). We hypothesized that the percent methylation levels within PMDs, which tend to be more variable than HMDs [20], and are distinct to placenta and pre-implantation embryos [21], might be sensitive as markers of early environmental exposures relevant to brain development.

Previous studies have demonstrated that placenta DNA methylation can provide specific signatures to early environmental exposures. Altered placental global or site-specific DNA methylation have been associated with exposures to: maternal cigarette smoking [22, 23]), bisphenol A [24], air pollution [25], and phthalates [26]. However, no study has yet investigated placental DNA methylation in relation to environmental contaminants using the unbiased whole genome bisulfite sequencing MethylC-seq approach which includes the analysis of all uniquely alignable CpGs in the genome.

To determine if common environmental exposures were associated with individual variation in placental DNA methylation, demographic, pregnancy, and exposure data from questionnaires given to MARBLES mothers were examined for associations by multivariate linear regression analysis with average PMD and HMD methylation levels, and the percentage of the genome within PMDs.

## Results

Figure 1 demonstrates the observation of bimodal global DNA methylation patterns characterized by the presence of HMDs and PMDs for MARBLES placental samples, similar to the three placental samples sequenced previously [21]. In this study population, mean average PMD methylation was 46.5% (SD = 2.6%) and mean average HMD methylation was 75.4% (SD = 0.9%), and the overall proportion of the genome in PMDs (with methylation <60%) was 43.7% (SD = 2.5%). Given that global genome-wide methylation patterns were very similar between placenta samples from 24 ASD and 23 typically developing (TD) children, primary analyses were conducted of the association between pregnancy and environmental exposures for all 47 placentas combined, though differential associations by ASD status were also considered. All results were similar when ASD status was added to the model to adjust for case status (Table S1), likely because ASD was not a significant predictor of HMD or PMD average methylation or the proportion of the genome as PMD. Also, we found that individuals with higher or lower methylation in HMDs/PMDs on one chromosome also tended to have similar higher or lower methylation in HMDs/PMDs on the other chromosomes. For this reason, exposures were examined in relation to average methylation over all PMDs and HMDs. Since a significant correlation between average PMD and average HMD methylation levels was observed within individuals, PMD analyses were conducted with and without adjustments for HMD, and *vice versa*. In previous analyses, sequencing run, order, and coverage, as well as child race/ethnicity and child sex were not significantly associated with PMD or HMD methylation, but were included here for consideration in multivariate models.

### Predictors of PMD Methylation

In bivariate analyses, maternal smoking history and professionally applied lawn and garden pesticides before or during

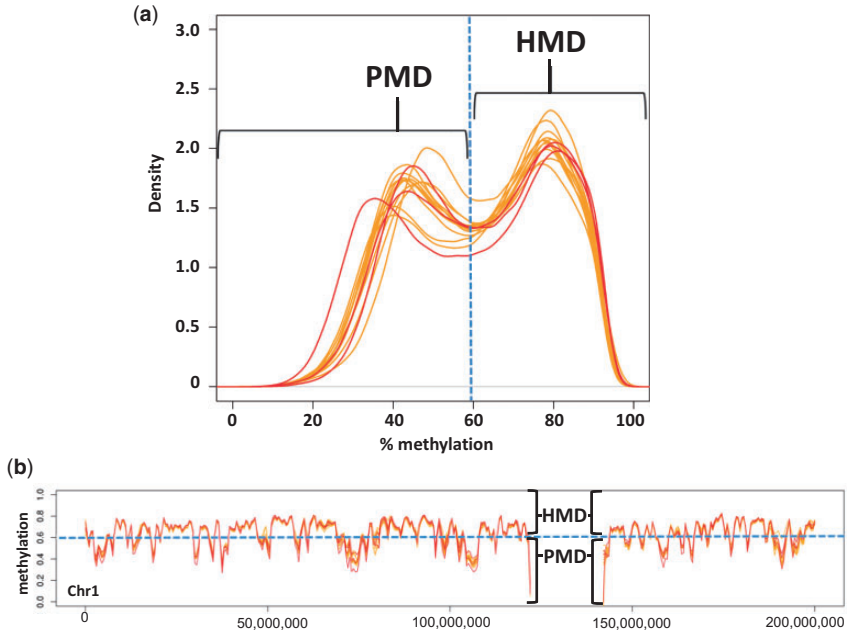


**Figure 1:** Determination of PMDs, HMDs, and percentage of genome in PMDs. **a** Individual histograms for 17 MARBLES placenta samples (orange histograms) as well as three biological replicates of "normal" non-MARBLES placentas (red histograms) with no known pathologies [20]. Methylation data were omitted if a window had fewer than 20 covered CpG sites. Blue vertical dotted line shows the 60% methylation cutoff between windows with low (PMD) and high (HMD) methylation. "Density" refers to the density distribution of the genomic windows sampled. **b** A representative example of individual PMDs and HMDs across chromosome 1, observed as sharp transitions between methylation states. The position of PMDs and HMDs is consistent between different placental samples, although more variation in percent methylation is observed over PMDs.

pregnancy were associated with higher placental PMD methylation (Table 1), there was a trend for higher PMD methylation in mothers with *MTFHR* 677 CT or TT genotypes, and a trend for lower PMD methylation in those exposed before or during pregnancy to flea and tick pouches used on pets. In analyses adjusting for HMD methylation, use of pet flea and tick pouches or any pet pesticides, and food folate in the second half of pregnancy were significantly associated with lower placental PMD methylation; there were trends towards associations with higher placental PMD methylation observed for mothers who ever smoked cigarettes. None of the other exposures examined were significantly associated with PMD methylation (Table 1).

In multivariate models selected based on the lowest AIC, mothers who reported pre-pregnancy or pregnancy exposure to any professionally applied lawn and garden pesticides (typically sprays and foggers) had higher placental DNA methylation across PMDs (Table 2). Lower placental DNA methylation across PMDs was observed in placentas of mothers who reported using pet pesticides including flea/tick pouches applied to pet, flea/tick collars, or flea/tick pet soaps/shampoos before or during pregnancy and who had non-white children (Table 2). After false discovery rate (FDR) correction, non-white race was no longer a statistically significant predictor, and pet pesticides were associated with a trend. In the subset with genotype data available, maternal *MTHFR* 677 CT/TT genotype was associated with higher PMD methylation after adjustment for other factors selected by best model fit (Table 2), and after adjustment for HMD methylation ($P = 0.01$), but was not significant after FDR correction (Table 2). In the HMD-adjusted model, only flea/tick pouches applied to pets before or during pregnancy remained statistically significant after FDR correction (Table 2). In post-hoc analyses, pet ownership was examined as a confounder of the association between pet pesticides and PMD methylation, and pet pesticides were assessed only within pet owners. The association between pet pesticides was attenuated when pet ownership was included in the model and in analyses restricted to pet owners, but there remained a trend towards an association between flea pouches and lower PMD methylation prior to FDR correction (Table S2); results were similar when adjusted for HMD methylation.

When predictors of PMD methylation were examined separately for ASD and TD children, professionally sprayed lawn and garden pesticides were a strong predictor in TD children, whereas maternal graduate education and flea pouches tended to be associated in both ASD and TD children (Tables S3). However, in TD children, pet ownership was also a strong predictor of lower PMD methylation, and when it was included in model selection and in analyses restricted to pet owners, flea pouches and pet pesticides were no longer associated with reduced PMD methylation.

## Predictors of HMD Methylation

In bivariate analyses, higher placental HMD methylation was predicted by maternal report of any professional exterminator-applied pesticides to her lawn or garden before or during pregnancy ($P = 0.01$), which became less than a trend after FDR correction ($P = 0.11$) and was not significantly associated after adjustment for PMD methylation (Table 3). After adjustment for PMD methylation, there was a trend for an association between higher HMD methylation and flea and tick pouch exposure before or during pregnancy ($P = 0.07$) and FFQ folate in the latter half of pregnancy ($P = 0.08$, Table 3), but these associations were not apparent after FDR-correction (Table 3). Pet ownership was not a significant predictor of HMD methylation (Table 3).

In multivariate models, higher placental HMD methylation was predicted by maternal report of prenatal exposure to any professionally applied lawn or garden pesticides before and

**Table 1:** Bivariate and HMD-adjusted associations between pregnancy exposures and placental PMD methylation

| Pregnancy exposure | N | Frequency exposed *n* (%) | Estimate (95% CI) | P-value | FDR P-value | Estimate (95% CI) adjusted for HMD methylation | P-value | FDR P-value |
|---|---|---|---|---|---|---|---|---|
| Own their home | 47 | 28 (59.6) | −0.11 (−0.42 to 0.20) | 0.48 | 0.99 | −0.05 (−0.29 to 0.2) | 0.71 | 0.99 |
| Maternal education | 47 | | | | | | | |
| Less than high school | | 20 (42.6) | 0.15 (−0.19 to 0.49) | 0.37 | 0.99 | 0.09 (−0.17 to 0.36) | 0.48 | 0.99 |
| Graduate or professional degree | | 8 (17.0) | 0.01 (−0.43 to 0.46) | 0.95 | 0.99 | −0.01 (−0.36 to 0.33) | 0.94 | 0.99 |
| Maternal height (m) | 47 | – | −1.04 (−3.06 to 0.98) | 0.31 | 0.99 | −0.53 (−2.12 to 1.05) | 0.50 | 0.99 |
| Maternal pre-pregnancy body mass index | 47 | | | | | | | |
| Overweight (25 to < 30) | | 14 (29.8) | 0.23 (−0.14 to 0.60) | 0.22 | 0.98 | 0.21 (−0.06 to 0.49) | 0.13 | 0.89 |
| Obese (30+) | | 13 (27.7) | 0.15 (−0.23 to 0.52) | 0.43 | 0.99 | 0.11 (−0.17 to 0.39) | 0.43 | 0.99 |
| Prenatal vitamin taken Pregnancy month 1 | 47 | 21 (44.7) | 0.02 (−0.29 to 0.33) | 0.89 | 0.99 | 0.09 (−0.15 to 0.33) | 0.45 | 0.99 |
| Mother ever smoked cigarettes | 46 | 20 (43.5) | 0.32 (0.03, 0.60) | **0.03** | 0.39 | 0.21 (−0.02 to 0.44) | ***0.08*** | 0.99 |
| Parity | 47 | – | −0.05 (−0.2, 0.10) | 0.53 | 0.99 | −0.01 (−0.13 to 0.11) | 0.90 | 0.99 |
| Gestational age (weeks) at delivery | 47 | – | 0.07 (−0.05 to 0.2) | 0.24 | 0.99 | 0.02 (−0.08 to 0.12) | 0.71 | 0.99 |
| Child birth weight (kg) | 39 | – | −0.01 (−0.39 to 0.37) | 0.95 | 0.99 | −0.14 (−0.44 to 0.17) | 0.37 | 0.99 |
| Family owned a cat or dog during pregnancy | 46 | 25 (54.4) | −0.29 (−0.58, −0.01) | **0.05** | 0.52 | −0.26 (−0.49, −0.04) | **0.02** | 0.31 |
| Pesticides applied in home during pregnancy | 44 | 10 (22.7) | −0.03 (−0.41 to 0.36) | 0.89 | 0.99 | −0.09 (−0.20 to 0.37) | 0.54 | 0.99 |
| Any professionally applied pesticides sprayed on lawn or garden before or during pregnancy | 45 | 12 (26.7) | 0.45 (0.14, 0.77) | **0.01** | 0.09 | 0.22 (−0.07, 0.51) | 0.14 | 0.91 |
| Used flea pouch products before or during pregnancy | 46 | 12 (26.1) | −0.34 (−0.69 to 0.01) | ***0.05*** | 0.59 | −0.35 (−0.61, −0.09) | **0.01** | 0.13 |
| Used any pet pesticides before or during pregnancy | 45 | 15 (33.3) | −0.26 (−0.57 to 0.06) | 0.11 | 0.84 | −0.25 (−0.50, −0.01) | **0.04** | 0.50 |
| Used any indoor pesticides during pregnancy | 47 | 10 (21.3) | −0.05 (−0.42 to 0.33) | 0.81 | 0.99 | 0.07 (−0.22, 0.37) | 0.63 | 0.99 |
| Maternal MTHFR677 CT or TT | 30 | 20 (67.7) | 0.26 (−0.03 to 0.56) | ***0.08*** | 0.74 | 0.15 (−0.11, 0.41) | 0.26 | 0.99 |
| Food folate (100 μg) from FFQ | | | | | | | | |
| FFQ 1 pregnancy weeks 1–20 | 28 | – | 0.03 (−0.17, 0.22) | 0.79 | 0.99 | 0.03 (−0.13 to 0.18) | 0.73 | 0.99 |
| FFQ 2 pregnancy weeks 21–40 | 20 | – | −0.09 (−0.28 to 0.1) | 0.32 | 0.99 | −0.13 (−0.25 to 0.00) | **0.05** | 0.55 |
| FFQ 1 if available, if not FFQ 2 | 33 | – | −0.02 (−0.21 to 0.16) | 0.79 | 0.99 | 0.01 (−0.13, 0.16) | 0.85 | 0.99 |
| Averaged Across FFQ 1 and FFQ 2 | 33 | – | −0.09 (−0.29, 0.12) | 0.39 | 0.99 | −0.08 (−0.23, 0.08) | 0.32 | 0.99 |

CI, confidence interval; FFQ, Food Frequency Questionnaire; OR, odds ratio. Bold p-value indicates significance at <0.05; Bold italic p-values are significant at <0.10.

after FDR correction ($P = 0.002$ and $P = 0.04$, respectively), but not after adjustment for PMD methylation (Table 2). After adjustment for PMD methylation, indoor pesticides sprayed in the home any time during pregnancy (reported in regularly collected home walk-through) was associated with a trend towards lower HMD methylation only prior to FDR correction.

Maternal dietary folate intake in the second half of pregnancy was associated with a trend towards higher HMD methylation in models adjusting only for PMD methylation ($P = 0.05$, Table 3) and also in multivariate models adjusting for other significantly variables selected by AIC (estimate per 100 μg = 0.18, 95% CI 0.03, 0.33, $P = 0.02$) in the subset of 20 with food frequency data, prior to but not after FDR corrections (Table 2).

When predictors of HMD methylation were examined separately for ASD and TD children, in-home and pet pesticides were predictors of HMD methylation only in children with ASD, but lawn and garden pesticides predicted HMD methylation in both ASD and TD children (Table S4). Child's birth weight was a predictor of higher HMD methylation in TD children only. There was also a trend for associations with lower HMD methylation by increasing sequencing run, order, and coverage. None of these associations persisted after FDR-correction.

## Predictors of PMD Genomic Coverage

The bimodal pattern of DNA methylation is observed from analysis of percent methylation over 20-kb windows, and <60% methylation is used as a determined cut-off for windows defined as PMDs. In bivariate analyses, prenatal lawn or garden pesticides were associated with lower PMD genomic coverage, and maternal smoking history was associated with a trend towards lower PMD coverage; neither of these associations was significant after FDR correction (Table S5). In multivariate models, identified predictors of the percent of 20-kb regions with methylation below 60% were similar to those for average PMD methylation levels, but associations with predictors were in the opposite direction, as expected, including a negative association with professionally applied lawn or garden pesticide exposure before or during pregnancy, and positive associations with non-white race and use of flea and tick pouches applied to pets before or during pregnancy (Table 2). Only the negative association with professionally applied lawn or garden pesticides remained statistically significant after FDR correction.

**Table 2:** Multivariable models of demographic and environmental predictors of placental methylation

| Placental methylation | Pregnancy exposure | Frequency exposed *n* (%) | Estimate (95% CI) | *P*-value | FDR *P*-value |
|---|---|---|---|---|---|
| PMD (*n*=44) | Lawn/garden pesticides[a] | 12 (27.3) | 0.45 (0.17, 0.72) | **0.002** | **0.03** |
| | Pet pesticides[a] | 15 (34.1) | −0.37 (−0.61, −0.12) | **0.004** | ***0.06*** |
| | Child non-White race | 16 (36.4) | −0.32 (−0.57, −0.07) | **0.01** | 0.18 |
| PMD in subset with maternal *MTHFR* 677 genotype (*n*=29) | Maternal *MTHFR* 677 CT or TT genotype | 20 (69.0) | 0.26 (0.05, 0.47) | **0.02** | 0.24 |
| | Lawn/garden pesticides [a] | 9 (31.0) | 0.24 (0.02, 0.47) | **0.04** | 0.44 |
| | Pet flea/tick pouches[a] | 9 (31.0) | −0.37 (−0.59, −0.15) | **0.002** | **0.04** |
| | Child non-White race | 9 (31.0) | −0.38 (−0.57, −0.19) | **0.0004** | **0.006** |
| | Maternal BMI 25+ [b] | 15 (51.7) | 0.20 (−0.004, 0.40) | ***0.05*** | 0.59 |
| PMD Adjusted for HMD (n = 45) | Pet flea/tick pouches [a] | 12 (26.7) | −0.36 (−0.60, −0.13) | **0.003** | **0.05** |
| | Child non-White race | 17 (37.8) | −0.20 (−0.40, −0.10) | **0.04** | 0.50 |
| | Mother ever smoked | 19 (42.2) | 0.19 (−0.01, 0.39) | ***0.06*** | 0.61 |
| | HMD methylation | – | 0.64 (0.42, 0.87) | **<0.0001** | **0.002** |
| PMD adjusted for HMD in subset with maternal *MTHFR* 677 genotype (*n* = 26) | Maternal *MTHFR* 677 CT or TT genotype | 17 (65.4) | 0.21 (0.05, 0.38) | **0.01** | 0.20 |
| | Pet flea/tick pouches [a] | 8 (30.8) | −0.36 (−0.56, −0.15) | **0.002** | **0.03** |
| | Sprays used in home [c] | 7 (26.9) | −0.15 (−0.30, 0.002) | ***0.05*** | 0.58 |
| | Child non-White race | 8 (30.8) | −0.26 (−0.43, −0.09) | **0.01** | 0.08 |
| | Maternal BMI 25+ [b] | 14 (53.9) | 0.27 (0.12, 0.42) | **0.001** | **0.02** |
| | HMD methylation | – | 0.36 (0.23, 0.49) | **<0.0001** | **0.002** |
| HMD (*n*=45) | Lawn/garden pesticides [a] | 12 (26.7) | 0.40 (0.16, 0.65) | **0.002** | **0.04** |
| | Assay run | – | 0.11 (−0.003, 0.23) | ***0.06*** | 0.60 |
| | Assay order | – | −0.03 (−0.06, −0.004) | **0.03** | 0.34 |
| HMD adjusted for PMD (*n*=44) | Sprays used in home [c] | 10 (22.7) | −0.17 (−0.33, 0.001) | ***0.05*** | 0.56 |
| | Assay order | – | −0.01 (−0.01, −0.002) | **0.01** | 0.12 |
| | PMD methylation | – | 0.56 (0.38, 0.74) | **<0.0001** | ***0.002*** |
| HMD adjusted for PMD in Subset with FFQ data (*n*=19) | FFQ dietary folate (100 μg/d) in late pregnancy | – | 0.18 (0.03, 0.33) | **0.02** | 0.28 |
| | Pet flea/tick pouches [a] | 7 (36.8) | −0.27 (−0.69, 0.15) | 0.19 | 0.97 |
| | Assay run | – | 0.23 (0.09, 0.37) | **0.004** | ***0.06*** |
| | Assay order | – | −0.04 (−0.08, −0.01) | **0.03** | 0.38 |
| | Assay coverage | – | −0.28 (−0.58, 0.02) | ***0.06*** | 0.649 |
| | PMD Methylation | – | 0.78 (0.51, 1.04) | **<0.0001** | **0.002** |
| Percent 20 kb regions with methylation < 60% (*n*=42) | Lawn/garden pesticides[a] | 10 (23.8) | −0.42 (−0.67, −0.17) | **0.002** | **0.03** |
| | Pet flea/tick pouches[c] | 9 (21.4) | 0.37 (0.03, 0.70) | **0.03** | 0.42 |
| | Sprays used in home [c] | 10 (23.8) | 0.30 (0.01, 0.58) | **0.04** | 0.44 |
| | Non-White race | 15 (35.7) | 0.33 (0.07, 0.58) | **0.01** | 0.19 |

[a]Includes exposure occurring in the 6-months before pregnancy.
[b]Pre-pregnancy body mass index of 25 or more (overweight or obese).
[c]Exposure occurring during pregnancy. Bold p-value indicates significance at <0.05; Bold italic p-values are significant at <0.10.

### Predictors of Methylation of Functional Gene Categories

In post-hoc analyses, we examined predictors of average methylation of three functional categories of regulatory elements based on placental chromatin state: promoters of active genes (TSSA), enhancers, and promoters of transcriptionally poised, bivalent genes (TssBiv) [27]. Active promoter methylation appeared more sensitive to assay parameters, primarily coverage, likely as a result of their small size. In multivariate models adjusted for coverage, maternal intake of prenatal vitamins in the first pregnancy month was significantly associated with increased active promoter methylation, but not after FDR corrections (Table S6). In the subset with food frequency data for the second half of pregnancy, food folate was associated with decreased active promoter methylation after adjustment for prenatal vitamin use in pregnancy month one and coverage. Average methylation at enhancers tended to be inversely associated with pet ownership prior to FDR correction (Table S7). Maternal *MTHFR* 677 CT or TT genotype was associated with increased average enhancer methylation prior to FDR correction, with and without adjustment for home ownership and low maternal education. Bivalent promoter methylation was associated with lawn and garden pesticides before and after adjustment for several other factors, but not after FDR correction (Table S8). In the subset with food frequency data for the second half of pregnancy, food folate was associated with decreased bivalent promoter methylation in the multivariate model prior to FDR correction, though the model was unstable given the small number of participants in this subset and the high number of variables selected by AIC.

## Discussion

We investigated the association between demographic, pregnancy, and environmental factors on placental methylation levels. Because placentation occurs early in human development, alterations in placental methylation levels may therefore reflect exposure effects on the earliest stages of pregnancy. Overall, most of the 16 examined pregnancy exposure variables

**Table 3:** Bivariate and PMD-adjusted associations between pregnancy exposures and placental HMD methylation

| Pregnancy exposure | N | Frequency exposed *n* (%) | Estimate (95% CI) | *P*-value | FDR *P*-value | Estimate (95% CI) adjusted for PMD methylation | *P*-value | FDR *P*-value |
|---|---|---|---|---|---|---|---|---|
| Own their home | 47 | 28 (59.6) | −0.09 (−0.35, 0.18) | 0.52 | 0.99 | −0.03 (−0.23, 0.18) | 0.80 | 0.99 |
| Maternal education | 47 | | | | | | | |
| Less than high school | | 20 (42.6) | 0.08 (−0.21, 0.37) | 0.58 | 0.99 | 0.00 (−0.23, 0.22) | 0.97 | 0.99 |
| Graduate or professional degree | | 8 (17.0) | 0.04 (−0.35, 0.42) | 0.85 | 0.99 | 0.03 (−0.27, 0.33) | 0.85 | 0.99 |
| Maternal height (m) | 47 | – | −0.68 (−2.40, 1.05) | 0.43 | 0.99 | −0.11 (−1.47, 1.25) | 0.87 | 0.99 |
| Maternal pre-pregnancy body mass index | 47 | | | | | | | |
| Overweight (25 to < 30) | | 14 (29.8) | 0.02 (−0.30, 0.35) | 0.90 | 0.99 | −0.11 (−0.36, 0.13) | 0.36 | 0.99 |
| Obese (30+) | | 13 (27.7) | 0.05 (−0.28, 0.38) | 0.77 | 0.99 | −0.04 (−0.29, 0.21) | 0.75 | 0.99 |
| Prenatal vitamin taken pregnancy month 1 | 47 | 21 (44.7) | −0.09 (−0.35, 0.17) | 0.49 | 0.99 | −0.10 (−0.30, 0.10) | 0.31 | 0.99 |
| Mother ever smoked cigarettes | 46 | 20 (43.5) | 0.16 (−0.10, 0.42) | 0.23 | 0.98 | −0.02 (−0.24, 0.21) | 0.89 | 0.99 |
| Parity | 47 | – | −0.05 (−0.18, 0.08) | 0.41 | 0.99 | −0.03 (−0.13, 0.07) | 0.59 | 0.99 |
| Gestational age (weeks) at delivery | 47 | – | 0.07 (−0.03, 0.18) | 0.17 | 0.95 | 0.03 (−0.05, 0.12) | 0.42 | 0.99 |
| Child birth weight (kg) | 39 | – | 0.15 (−0.13, 0.44) | 0.29 | 0.99 | 0.16 (−0.07, 0.39) | 0.17 | 0.95 |
| Family owned a cat or dog during pregnancy | 46 | 25 (54.4) | −0.05 (−0.31, 0.22) | 0.73 | 0.99 | 0.13 (−0.09, 0.43) | 0.24 | 0.99 |
| Professionally applied pesticides sprayed in home during pregnancy | 44 | 10 (22.7) | −0.13 (−0.43, 0.18) | 0.41 | 0.99 | −0.11 (−0.33, 0.11) | 0.31 | 0.99 |
| Any professionally applied pesticides sprayed on lawn or garden before or during pregnancy | 45 | 12 (26.7) | 0.39 (0.11, 0.66) | **0.01** | 0.11 | 0.17 (−0.09, 0.43) | 0.19 | 0.97 |
| Used flea pouch products before or during pregnancy | 46 | 12 (26.1) | 0.02 (−0.29, 0.32) | 0.92 | 0.99 | 0.22 (−0.02, 0.46) | ***0.07*** | 0.69 |
| Used any pet pesticides before or during pregnancy | 45 | 15 (33.3) | −0.01 (−0.29, 0.28) | 0.97 | 0.99 | 0.14 (−0.08, 0.37) | 0.21 | 0.98 |
| Used any indoor pesticides during pregnancy | 47 | 10 (21.3) | −0.15 (−0.47, 0.17) | 0.34 | 0.99 | −0.13 (−0.37, 0.12) | 0.31 | 0.99 |
| Maternal MTHFR677 CT or TT | 30 | 20 (67.7) | 0.26 (−0.11, 0.62) | 0.16 | 0.94 | 0.08 (−0.25, 0.40) | 0.63 | 0.99 |
| Food folate (100 μg) from FFQ | | | | | | | | |
| FFQ 1 pregnancy weeks 1–20 | 28 | – | 0.00 (−0.16, 0.16) | 0.99 | 0.99 | −0.01 (−0.14, 0.11) | 0.83 | 0.99 |
| FFQ 2 pregnancy weeks 21–40 | 20 | – | 0.04 (−0.14, 0.23) | 0.63 | 0.99 | 0.12 (−0.01, 0.24) | 0.08 | 0.71 |
| FFQ 1 if available, if not FFQ 2 | 33 | – | −0.05 (−0.20, 0.11) | 0.54 | 0.99 | −0.03 (−0.15, 0.08) | 0.56 | 0.99 |
| Averaged across FFQ 1 and FFQ 2 | 33 | – | −0.01 (−0.19, 0.16) | 0.88 | 0.99 | 0.04 (−0.10, 0.17) | 0.58 | 0.99 |

CI, confidence interval; FFQ, Food Frequency Questionnaire; OR, odds ratio. Bold p-value indicates significance at <0.05; Bold italic p-values are significant at <0.10.

examined were not predictive of average placental PMD or HMD methylation or the genomic proportion of PMDs. There were associations between methylation and dietary folate and *MTHFR*677 CT or TT (less efficient folate metabolizing) genotypes in the subsets with these data, but these findings were not consistent and were relatively unstable. Self-reported exposure during pregnancy to various types of pesticides, especially sprays and foggers professionally applied to lawns and gardens and those applied to control fleas and ticks on pets, were the most significant predictors of each type of methylation measure. These associations between pesticide exposures and placental methylation varied in direction across pesticide application types. This could suggest that each pesticide application type has differential effects on methylation, or this could be taken as evidence of chance findings with many comparisons within a relatively small sample, despite correction for multiple comparisons.

The negative association between PMD methylation and pet or flea/tick pesticides was attenuated to non-significance after FDR correction with adjustment for pet ownership. This suggests that there are epigenetic changes associated with having a pet that partially explained the association with pet pesticides, and is consistent with the inverse correlation of pet ownership with enhancer methylation we observed prior to FDR correction. These results are biologically plausible given known associations between early life exposure to pets in the home and other health-relevant adaptations, including development of more diverse infant fecal microbial composition [28], differences in innate [29], and adaptive [30] immune systems, and fewer atopic diseases in the child [31–33]. It is also possible this observed association could have been a chance finding as a result of multiple comparisons that was lost after adjustment.

Lawn and garden pesticides tended to be robustly associated with higher placenta PMD methylation in children with both ASD and TD. Though maternal report of pesticide exposures was obtained prospectively, has been shown to be highly reliable [34], and correlates with measured pesticide exposures found in dust samples [35], inaccuracies in maternal reported pesticide exposure could have attenuated associations. Further, because these factors were examined in relation to the percent methylation averaged across PMDs/HMDs throughout the genome, effects at individual domains or genes could have been diluted. However, the additional positive association observed with lawn and garden pesticides and methylation over poised, bivalent promoters after multiple adjustments further suggests the meaningful association of these results.

Another potential limitation of this study was that the heterogeneity of placenta samples might have obscured inter-individual differences, despite efforts to collect samples from a uniform manner and location. The samples analyzed were a heterogeneous mixture of cell types, including trophoblasts, mesenchymal stromal cells, and fetal vascular and hematopoetic cells. However, we previously observed no detectable differences in large scale methylation levels between human placentas isolated from 10 different regions (including the maternal and fetal side) [20] or between rhesus trophoblast compared with rhesus placenta [19]. Therefore, at least for placenta, cell type and regional differences are unlikely to be a major factor for large-scale methylation levels observed in this study.

Finally, pesticide classes with differential mechanisms of action were not distinguished in this study; future studies should more carefully examine specific pesticide classes in relation to DNA methylation to determine which have the strongest associations. The majority of pesticides used in the reported household products are pyrethroids since the ban of organophosphate pesticides [36–38]. The associations with pet pesticides were attenuated, but still present when restricted to pet owners. The strongest and most robust finding was for an association between sprays and foggers professionally applied outside the home and higher average methylation within PMDs and a reduced proportion of the genome in PMDs. Formulations for pesticides used outdoors in professional lawn and garden applications (e.g., Bifenthrin) are typically designed to be more stable in sunlight and degrade less rapidly [39]; they also show greater neurotoxicity [40, 41]). The association between these outdoor pesticides and differential DNA methylation is congruent with other evidence for *in vitro* [42] and *in vivo* [43–45]) effects of pesticides on DNA methylation.

What biological insights can we infer from these large-scale genome-wide methylation differences associated with pesticides? Because DNA methylation patterns, including those of PMDs and HMDs, mirror the transcriptional patterns in placenta and pre-implantation embryos [19], gene expression changes induced by pesticides could be reflected in the levels of placental methylation. Since PMDs reflect genes that are inactive transcriptionally, but environmentally responsive, they may reflect a type of chromatin "storage boxes" within the genome in early life [46]. Differences in the average PMD methylation levels genome-wide may reflect the extent to which these "storage box genes" were transcribed or utilized *in utero*. For instance, both PMD and related poised, bivalent promoter methylation [20] were associated with lawn or garden pesticide use, suggesting past transcriptional differences in placenta associated with pesticide exposure. Though average PMD or HMD methylation levels were not associated with ASD in this study, it is possible that global changes across PMDs/HMDs linked to exposures are associated with more specific methylation changes that influence ASD risk in a subset. Further, perhaps these methylation changes alone are not enough to induce ASD, but are but one hit in a multi-hit process. Finally, these exposure markers could be associated with adverse outcomes other than ASD. These findings should be explored further in larger studies with biomarkers for pesticide exposures.

This study provides some of the first evidence on pregnancy and environmental factors that could influence DNA methylation in placental tissue of children at high risk for ASD. We found consistent associations of maternal PMD and HMD placental DNA methylation with outdoor pesticides, suggesting a possible mechanism by which pesticide exposures in prenatal life could alter gene expression and its sequelae. Larger studies using more detailed measures and biomarkers of pesticide exposure should attempt to confirm these associations. More generally, MethylC-seq analyses of human placenta samples could be useful in future prospective studies for the detection of exposure relevant methylation biomarkers.

## Methods

### MARBLES Study and Sample Selection

Placenta tissues were obtained as previously described [47] from the MARBLES (**M**arkers of **A**utism **R**isk in **B**abies: **L**earning **E**arly **S**igns) study, a prospective study of the environmental, genetic, and epigenetic factors leading to autism. Parents were enrolled that already had a child with autism and were pregnant or planning another pregnancy. The younger sibling from this pregnancy was at significantly (13-fold) higher risk of having autism than children in the general population [10]. MARBLES recruited families from Northern California from lists of children receiving services through the California Department of Developmental Services, from other studies at the MIND Institute, and by self-referral. Inclusion criteria for the MARBLES Study included: (i) maternal age at least 18 years; (ii) planning a pregnancy or pregnant; (iii) one or both parents of the current or planned pregnancy being the biological parent of a child with ASD; and (iv) residence within the specified catchment area in California; (v) English-speaking. Proband ASD status was confirmed and mothers were seen at regular intervals during pregnancy with biosample collection starting at enrollment and continuing at each visit. The placenta, cord blood, and other samples were collected and frozen at birth and the children were followed until a diagnosis of autism or TD was obtained at age 36 months by ADI-R in addition to other assessments. Once placentas were obtained shortly after birth, they were chilled, transported to a central lab and maintained until processing, with a time interval between birth and processing of 1–24 h. 3 × 3 cm full thickness samples were taken from a uniform location mid-way between the cord insertion and periphery, assigned sample identifiers, and were stored frozen at −80°C. For the purposes of this investigation, placenta specimens were removed from the freezer and, while still frozen, aliquots were taken from the fetal surface just beneath the chorioamniotic membranes, moved to a separate container and refrozen. Batched aliquots were thawed, whole aliquot specimens were homogenized, and DNA was isolated using Qiagen's Puregene kit from MARBLES placenta samples from full-term pregnancies of 24 children who received a final 36-month clinical diagnosis of ASD by 29 September 2014 and 23 typically developing children matched to the children with ASD by gender and birth year (within 1.5 years), with preference given to those with greater availability of other study data. Due to the naturally occurring high proportion of males to females with autism, seen also in the MARBLES study, only two each of the autism and typical placentas were derived from births of female neonates. All participants gave written informed consent for data and sample use and protocols were approved by UC Davis IRB.

### Diagnostic Classification

Children's development was assessed by trained and reliable examiners with final diagnostic assessments at 36 months. All children are assessed for autism symptoms using the gold

standard Autism Diagnostic Observation Schedule-Generic (ADOS) ([48, 49]). A best estimate clinical diagnosis (BECD) was given through consensus of two clinicians based on *DSM-IV* or *DSM-5* criteria. Placenta samples were categorized based on whether the child met ADOS and *DSM* criteria for ASD, showed TD, or had impairments in some domains, but did not meet the full ASD diagnostic criteria (other developmental concerns).

### MethylC-seq

Placenta samples were frozen immediately after birth. As previously described [20], 5-μg placental DNA was extracted, sonicated to ~300 bp, and methylated Illumina adapters were ligated to the ends using NEB's NEBNext DNA library prep kit. The library was bisulfite converted using Zymo's EZ DNA Methylation-Lightning Kit, amplified for 14 cycles using PfuTurbo Cx, purified with Agencourt AMPure XP beads, and sequenced on an Illumina HiSeq 2000. Laboratory personnel were blinded to the case status of the samples, and samples were arranged randomly. Reads were mapped to the hg19 version of the human genome using BS Seeker [50]. To prevent clonal PCR amplification biases, only one read out of those with identical genomic positions was kept. CpG site methylation data were combined from both DNA strands. Sequencing information for each placenta sample, including the total reads, total mapped reads, coverage, and conversion efficiency is provided in Table S9.

### Defining PMDs/HMDs

PMDs and HMDs were defined as previously described [20]. In brief, methylation data from 17 typical placentas were combined to create a single, high-coverage map of methylation across the genome (as in Fig. 1). In tracks of percent methylation in the UCSC Genome Browser and placental tracks available through the public track hub, "UCD Methylation" (http://genome.ucsc.edu), PMDs and HMDs can be detected visually as large domains of lower levels of DNA methylation. Visually annotated PMD and HMD portions of this consensus genome were used to train a two-state hidden Markov model (HMM) to differentiate PMDs and HMDs using StochHMM [20, 51]. The model was then applied to the same high-coverage methylation data to define the boundaries of PMD/HMDs in typical placentas. Those boundary coordinates were used for calculating average percent methylation over all PMDs and all HMDs in each MARBLES placenta sample.

### Defining Enhancers, Active Promoters, and Poised Promoters from Chromatin State Maps

Previously described chromatin state HMM analysis in placenta (ChromHMM) [27] were used to define active promoters (ChromHMM state 1, TssA, defined by H3K4me3), enhancers (ChromHMM state 7, Enh, defined by H3K4me1), and bivalent promoters (ChromHMM state 10, defined by H3K4me3 and H3K27me3). For each of these placental ChromHMM states, percent methylation was calculated for each instance of that state in the genome by averaging over the CpG sites in the element and then averaging over all the element of that state in the genome. Mean percent methylation of ChromHMM-defined states is shown for ASD and TD placenta samples in Table S10.

### Statistical Analyses for Associations between Methylation and Demographic Characteristics and Environmental Exposures

Information on demographic factors, environmental exposures, nutritional supplement use, and medical history were prospectively collected through telephone-assisted interviews, mailed questionnaires, and medical record abstraction. The following variables were analyzed for associations with PMD total average methylation, HMD total average methylation, and the percent of the 20-kb windows with methylation below 60%: sequencing run, order, and coverage; child race (white non-Hispanic [reference], Asian, multi-racial, white Hispanic, non-white Hispanic); child sex (male [reference]/female); maternal pre-pregnancy body mass index (BMI) (categorized as <25 [reference], 25 to <30 [overweight], and 30+ [obese]), home ownership (yes/no [reference]); whether the mother ever smoked in her lifetime (yes/no [reference]); maternal education (no college degree, college 4-year degree [reference], graduate or professional degree); gestational age of child (in days); parity (one previous live birth is reference); maternal height; child birthweight (grams); professionally applied pesticides to lawn or garden 6 months before pregnancy through the end of pregnancy (yes, no [reference]); professionally applied pesticides to inside of home before or during pregnancy (yes, no [reference]; flea/tick pouches applied to pet, flea/tick collars, or flea/tick pet soaps or shampoos before or during pregnancy (yes, no [reference]). Many exposure categories were collapsed for inclusion in multivariate models as appropriate based on bivariate effect estimates and AIC. Because only one mother had underweight pre-pregnancy BMI (<18.5) or reported smoking during pregnancy, and none reported not taking a prenatal vitamin at any time during pregnancy, we were unable to evaluate these exposures in relation to methylation. Maternal *MTHFR* 677 genotype from Taqman assays in maternal blood DNA and dietary folate equivalents calculated from Block food frequencies completed by mothers for the first half and second half of pregnancy were also examined in bivariate analyses (Tables 1, 3, S2). Because these data were only available for a subset of those with methylation, these variables were not considered in multivariate model selection, but if they were significant at <30% then they were examined in separate adjusted models (Table 2).

We first performed bivariate linear regression using SAS software version 9.4 for each exposure in relation to PMD methylation and separately to HMD methylation (Tables 1, 3, S2). To facilitate interpretation of regression coefficients, PMD and HMD methylation was expressed in 5 percentage point and 2 percentage point units, respectively, so that coefficients (and effect size estimates based on them) correspond to an approximately 2 SD change in the dependent variable. Each exposure that was associated with methylation at $P < 0.30$ in the bivariate analysis was then retained in a full multivariate model. Subsequently we used Akaike information criterion (AIC) to select the model that balances best fit and parsimony among candidate models [52, 53]. To produce our final models, we used sandwich estimators to produce homoscadisticity-robust estimates of the 95% confidence interval given that the conditional variance of outcomes appeared to vary with the regression-based predicted values. We assessed predictors of PMD methylation, PMD methylation adjusted for HMD methylation, HMD methylation, HMD methylation adjusted for PMD methylation and the percent of the 20-kb windows with methylation below 60% [using a 6% (2 SD) change as the unit]. To account for the multiplicity of hypotheses being assessed, we adjusted the

P-values to control the FDR at 5% [54]. Once significant factors were identified, diagnostic status was added to the model to examine the adjusted association between ASD and methylation. In post-hoc analyses, predictors of average methylation across other functional categories were also examined, including: promotors of active genes, enhancers, and promoters of transcriptionally poised, bivalent genes.

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## Data Sharing

Sequencing data are available in NDAR #362 for those participants who consented to data release.

## Supplementary data

Supplementary data are available at *EnvEpig* online.

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